ISOLATION OF BUTYRATE-UTILIZING BACTERIA FROM THERMOPHILIC AND MESOPHILIC METHANE-PRODUCING ECOSYSTEMS

BY

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Abstract of Dissertation Presented to the Graduate School
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The ability of various ecosystems to convert butyrate to methane was studied in order to isolate the bacteria responsible for the conversion. When thermophilic digester sludge was enriched with butyrate, methane was produced without a lag period. Marine sediments enriched with butyrate required a 2-week incubation period before methanogenesis began. When hypersaline sediments were enriched with butyrate, methane was not produced after 3 months. A thermophilic digester was studied in more detail and found by most-probable-number enumeration to have ca. 5×10^6 butyrate-utilizing bacteria/ml of sludge. A thermophilic butyrate-utilizing bacterium was isolated in coculture with Methanobacterium thermoautotrophicum and a Methanosarcina sp. This bacterium was a gram-negative, slightly curved rod that occurred singly, was nonmotile, and did not appear to produce spores. When this coculture was incubated with Methanospirillum hungatei at 37°C, the quantity of methane produced was less than 5% of that produced when the

coculture was incubated at 55°C, the routine incubation temperature. The coculture required clarified digester fluid (CDF), which could not be replaced by rumen fluid (RF). The addition of yeast extract to a medium containing 5% CDF stimulated methane production when the Methanosarcina sp. was present. Hydrogen in the gas phase prevented butyrate utilization. However, when the hydrogen was removed, butyrate utilization began. Penicillin G and D-cycloserine caused the complete inhibition of butyrate utilization by the coculture.

The thermophilic digester was infused with butyrate at the rate of 10 μmoles/ml of sludge per day. Biogas production increased by 150%, with the percentage of methane increasing from 58% to 68%. Acetate, propionate, and butyrate did not accumulate. When the infusion rate was increased to 20 μmoles/ml of sludge per day, an unstable digestion resulted.

Butyrate-utilizing enrichments from mesophilic ecosystems were used in obtaining cocultures of butyrate-utilizing bacteria. These cocultures served as inocula for attempts to isolate pure cultures of butyrate-utilizing bacteria by use of hydrogenase-containing membrane fragments of Escherchia coli. After a 3-week incubation period, colonies appeared only in inoculated tubes that contained membrane fragments and butyrate.

INTRODUCTION

In anaerobic ecosystems where light, sulfate, and nitrate are absent, such as digesters and freshwater sediments, organic matter is degraded exclusively to methane and carbon dioxide. This degradation is mediated by several groups of bacteria and is regulated by the hydrogen concentration (21,29). Hydrogen does not accumulate and is difficult to detect in methane-producing ecosystems because of rapid interspecies hydrogen transfer. Interspecies hydrogen transfer is the utilization of hydrogen by one bacterial species (hydrogenotroph) that is produced by another bacterial species (hydrogenogen) (33,34). This phrase was introduced by Iannotti et al. (15) when they compared the fermentation products formed by a pure culture of Ruminococcus albus with the fermentation products formed when this organism was grown with a hydrogenotroph. R. albus is a carbohydrate-fermenting organism that produces acetate, ethanol, and hydrogen when it is grown in continuous culture with glucose as the sole carbon and energy source. However, when R. albus is grown in a mixed continuous culture with Vibrio succinogenes, a hydrogenotroph, the only products detected are acetate and succinate. Succinate is formed when V. succinogenes uses the hydrogen produced by R. albus to reduce fumarate which R. albus alone cannot use as an electron acceptor. Thus, because of the interspecies transfer of hydrogen from R. albus to V. succinogenes, there is a shift

in fermentation products from the less reduced ethanol to the more reduced acetate and hydrogen.

Selenomonas ruminantium is a carbohydrate-fermenting organism that when grown in pure culture produces trace amounts of hydrogen (27). Hydrogen production, however, as indicated by the amount of methane formed, is stimulated almost 100-fold when cocultured with methanogenic bacteria (27). Subsequent analysis (9) shows that the fermentation products formed from glucose are altered by the presence of hydrogen-utilizing methanogens. The electron sink fermentation products, lactate and propionate, are produced in decreased amounts, whereas acetate and hydrogen formation increases. It has been suggested (9) that the production of hydrogen results from reduced nicotinamide adenine dinucleotide (NADH); thus hydrogen becomes a major electron sink product and alters the fermentation products formed.

Similar results are observed when cellulose is fermented by Ruminococcus flavefacians (16). In the presence of Methanobacterium ruminantium, a hydrogenotropic methanogen, major fermentation products shift from succinate and acetate to acetate and hydrogen, which is evidenced by the large amounts of methane formed.

These studies confirm Hungate's hypothesis (13) that methanogenesis in the rumen provides for the removal of electrons from pyruvate via the formation of hydrogen. This hypothesis arose from observations that, when many rumen bacteria are grown in pure culture, a variety of products are found that are not found in the rumen. These products are electron sink products that result from the oxidation of NADH. The removal of electrons released by catabolism via hydrogen formation

results in the net removal of electrons from the rumen because the methanogenic bacteria maintain a low partial pressure of hydrogen by using it to reduce carbon dioxide to methane. The methane then leaves the rumen when the ruminent eructates.

All of the fermentative bacteria described above, as well as other fermentative bacteria, are able to produce alternative electron sink products in place of hydrogen when the partial pressure (pH_2) of hydrogen is not maintained sufficiently low for oxidation of NADH. Table 1 illustrates the required lowered pH_2 to oxidize NADH. Formation of H_2 from NADH, when products and reactants are equimolar, will not occur as indicated by the positive $\Delta G^{\circ\prime}$ for the reaction. As the concentration of H_2 approaches zero, the $\Delta G^{\circ\prime}$ for the reaction will become negative. If all the electrons carried by NADH are ultimately used to form CH_4 via H_2, as in the summation reactions in Table 1, the overall reaction has a $\Delta G^{\circ\prime}$ of -63.6 kJ. Thus, alternate electron sink products are not formed.

There are, however, other hydrogen-producing bacteria whose growth is dependent upon the constant removal of the hydrogen produced by the oxidation of NADH. Being unable to produce alternate electron sink products, these bacteria are obligate hydrogenogens. They are involved in the utilization of ethanol (8), propionate (5), butyrate (22,23), other fatty acids (23), benzoate (11,26), and possibly other substrates.

Methanobacillus omelianskii has been described by Barker (4) as being able to convert ethanol and carbon dioxide to acetate and methane. In 1967, Bryant et al. (8) were able to separate cultures of M. omelianskii into two bacterial species. Their studies reveal

Table 1. Oxidation of reduced nicotinamide adenine dinucleotide (NADH) by removal of H_2 via methane formation

Reaction	$\Delta G°'$ (kJ/reaction)[a]
1. $NADH + H^+ \rightarrow H_2 + NAD^+$	+18.0
2. $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
Summation:	
$4NADH + 4H^+ \rightarrow 4H_2 + 4NAD^+$	+72.0
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
$4NADH + HCO_3^- + 5H^+ \rightarrow 4NAD^+ + CH_4 + 3H_2O$	-63.6

[a]Values taken from or calculated from data in Thauer et al. (31).

that the presence of the two separate bacteria are necessary for the conversion to occur. One of the two bacteria, the S organism, oxidizes ethanol with the production of acetate and H_2, and will not grow alone. The second species is a methanogenic bacterium which utilizes H_2 but not ethanol for growth and methane production. Bryant et al.(8) also proposed that fatty acids other than formate and acetate are anaerobically degraded by nonmethanogenic bacteria similar to the S organism with the production of H_2.

As is the case for the oxidation of NADH described above, the pH_2 must be maintained at a low level for the oxidation of ethanol to occur in the coculture just described. Shown in Table 2 are the equations and free-energy changes for the oxidations of ethanol as well as propionate and butyrate. At equimolar concentrations of products and reactants, propionate has the highest $\Delta G^{\circ\prime}$: +76.1 kJ/reaction. Butyrate is intermediate with a +48.1 kJ/reaction, whereas ethanol is the lowest with a +19.2 kJ/reaction. When the concentrations of products are lowered, as by the removal of H_2, the reactions have a negative $\Delta G^{\circ\prime}$ and, hence, become thermodynamically possible. The most difficult reaction to make possible via H_2 removal is the oxidation of propionate; the reaction is less difficult with butyrate and least difficult with ethanol. The pH_2 must be 10^{-6}, 10^{-5}, and 10^{-3} atm., respectively (21). The separation of Methanobacillus omelianskii cultures into two components (8) was an important contribution to the understanding of microbial interactions in the fermentation of organic matter to methane because it gave initial insight into the physiological dependence of one group of bacteria on another group of bacteria.

Table 2. Equations and free-energy changes for oxidation of ethanol, propionate, and butyrate

Reaction	$\Delta G^{\circ\prime}$ (kJ/reaction)[a]
1. $CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2H^+$	+19.2
2. $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$	+76.1
3. $CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$	+48.1

[a]Values taken from or calculated from data in Thauer et al. (31).

Smith's report (29) summarized the results obtained when domestic sludge was enriched with propionate or butyrate. When these enrichments were rapidly sparged, H_2 was detected, indicating the presence of a physiological group of bacteria that produces H_2 from propionate or butyrate. In nonsparged enrichments, H_2 was not detected because of the interspecies transfer of H_2 to the methanogens and the subsequent production of CH_4. Both enrichments utilized H_2-CO_2 without a lag, indicating the existence of a population of hydrogenotrophs. Propionate or butyrate utilization was inhibited by the presence of H_2, indicating that removal of H_2 must take place in order for utilization to occur.

The first report of a fatty acid-utilizing bacterium is that of an anaerobic bacterium that utilizes butyrate as well as other fatty acids (23). This organism, which has subsequently been named Syntrophomonas wolfei (22), β-oxidizes saturated fatty acids (butyrate through octanoate) to acetate and H_2 or to acetate, propionate, and H_2. S. wolfei is a gram-negative helical rod with laterally inserted flagella and a sluggish twitching motility (23). When S. wolfei is cocultured with a Desulfovibrio sp., a generation time of 54 hours is obtained, which is less than the generation time of 87 hours when it is cocultured with M. hungatei. Compounds tested and found not to act as electron acceptors are dimethyl sulfoxide, fumarate, malate, nitrate, oxygen, sulfate, sulfite, sulfur, and thiosulfate (23). In addition, manganese oxide, methyl viologen, palladium chloride, phenosafranin, tetrazolium chloride, and trimethylamine-N-oxide are not utilized as electron acceptors when butyrate is the electron donor (22). The presence of 80% H_2 in the gas phase inhibits growth and butyrate utilization when S. wolfei is

cocultured with M. hungatei (22). S. wolfei did not grow alone when the gas phase was continually recycled through hot copper oxide filings to remove H_2 (22). Cells contained peptidoglycan and poly-β-hydroxybutyrate (22). A morphologically similar bacterium was isolated from rumen fluid (24).

Syntrophobacter wolinii is a nonmotile, gram-negative rod that degrades propionate, but not other fatty acids, to acetate and, presumably, H_2 and CO_2 (or formate) only in the presence of a hydrogenotrophic bacterium (5). When S. wolinii is cocultured with a Desulfovibrio sp., the hydrogenotroph, the doubling time is about 87 hours. The doubling time increases to 161 hours when the Desulfovibrio sp. is present as a minor component of the coculture and Methanospirillum hungatei is the major hydrogenotrophic organism. S. wolinii is a strict anaerobe.

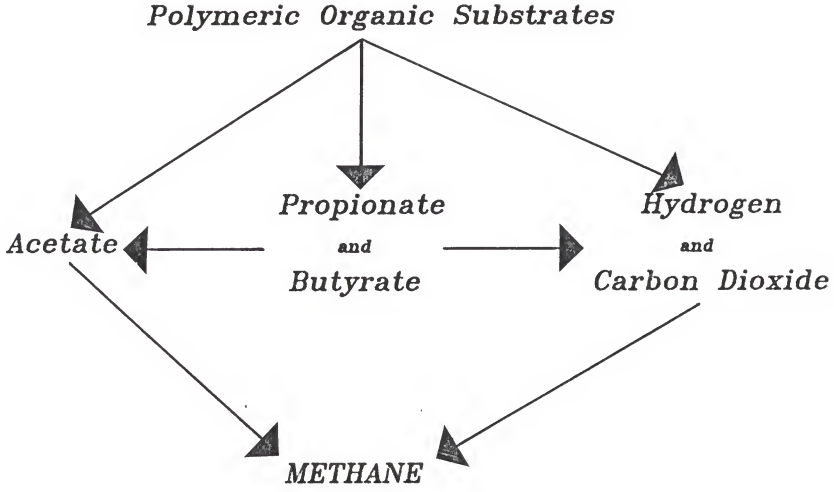
Another example of an obligate hydrogenogenic bacterium is one that does not utilize fatty acids. Benzoate is degraded to acetate and, presumably, CO_2 and H_2 (or formate) by a gram-negative, motile, rod-shaped bacterium in coculture with the hydrogenotrophic Desulfovibrio sp. (26). The benzoate utilizer does not use other common aromatic compounds, C_3-C_7 monocarboxylic acids, or C_4-C_6 dicarboxylic acids for growth. It is unable to use nitrate, sulfate, or fumarate as alternate electron acceptors. In coculture with the Desulfovibrio sp., the generation time is 132 hours, whereas in coculture with M. hungatei, the generation time is 166 hours. The presence of 80% H_2 in the gas phase inhibits utilization of benzoate.

Descriptions of the two species of fatty acid-utilizing, hydrogenogenic bacteria are the only information available concerning the

bacteria involved in the degradation of fatty acids in the fermentation of organic matter to methane. There are at least three major groups of bacteria involved in the fermentation of the polymeric substrates, cellulose, starch, protein, lipids, etc., to methane (6,18,21,29). The methane fermentation is divided into three distinct stages (6,21,29), as shown in Figure 1. Fermentative bacteria are the first of the three major groups to react. This group is a complex mixture of many obligate and facultative anaerobic bacterial species (12,21) which act on the polymeric substrates. Polysaccharides are initially hydrolyzed to their component sugars, which are then transported into the fermentative bacteria. The sugars are fermented primarily by the Embden-Meyerhof-Parnas pathway with the production of a variety of electron sink products such as acetate, propionate, and butyrate (6), as is shown in Figure 1. Proteins are hydrolyzed to amino acids which are then fermented to the products shown in Figure 1, in addition to other products such as isobutyrate, phenylacetate, and phenylpropionate (6). Lipids are hydrolyzed to glycerol, a fermentable substrate, and long-chain fatty acids which are not fermented by the fermentative bacteria (6). The fermentative bacteria have been studied in detail, but additional research is needed.

The terminal group of bacteria, the methanogens, is generally understood although not as well as the fermentative bacteria. These bacteria are essential to the complete anaerobic degradation of organic matter. Otherwise, organic acids that contain about as much energy as the polymeric substrates would accumulate and anaerobic degradation would cease (6,21). As shown in Figure 1, these unique bacteria utilize

Figure 1. Schematic representation of the three stages of anaerobic degradation of organic matter to methane.


Polymeric Organic Substrates
Propionate
and
Butyrate
Acetate
Hydrogen
and
Carbon Dioxide
METHANE

a limited number of substrates, mainly acetate and H_2-CO_2, to produce methane, which contains 90% of the energy of the polymeric substrates (6,21).

The third group of bacteria is the least understood of the three groups. These bacteria, the hydrogenogens, utilize the fermentation products of the first group and produce substrates that are utilizable by the methanogenic group. In Figure 1, this reaction is shown by the degradation of propionate and butyrate to acetate and H_2-CO_2. The primary reason for the lack of information concerning these bacteria is the difficulty in isolating and manipulating them. Existing studies are of cocultures because the bacteria have not been isolated in an axenic culture.

The hydrogenogens are obligate proton-reducing bacteria and produce H_2 with the concomitant oxidation of NADH (21). Therefore, the enzyme hydrogenase may be useful in the isolation of axenic cutlures of hydrogenogens. Hydrogenase is the collective term for enzymes which catalyze the reversible reaction (1,28):

$$H_2 \rightleftarrows 2H^+ + 2e^-$$

Thus, a cell-free extract containing hydrogenase, and other components necessary to transfer electrons from H_2 to an electron acceptor, may make possible the isolation of axenic cultures of hydrogenogenic bacteria.

The utilization of fatty acids may be the rate-restricting step in the fermentation of organic matter to methane (19,21). The bacteria responsible for these reactions have not been well studied. Therefore,

to better exploit the production of methane from biomass, these bacteria must be better understood. It is the objective of this study to increase the understanding of the fatty acid-utilizing bacteria by attempting to isolate cocultures of new species and to attempt the isolation of pure cultures of these bacteria. The knowledge gained, it is hoped, may be used to bring about the more efficient degradation of biomass to methane.

MATERIALS AND METHODS

Organisms, Media, and Growth Conditions

Methanobacterium thermoautotrophicum strain ΔH, Methanospirillum hungatei strain JF-1, and Escherichia coli ATCC 11303 were obtained from our culture collection. Desulfovibrio sp. strain G-11 was a gift gratefully received from Dr. Dave Boone.

Mb. thermoautotrophicum was incubated at 55°C in medium number 2 of Balch et al. (3). Ms. hungatei was incubated at 37°C in medium number 1 of Balch et al. (3). To prepare these media, stock solutions of trace minerals, trace vitamins, and various salt solutions were prepared and frozen until used. The stock solution of trace minerals contained in grams per 100 ml of distilled water, the following: nitrilotriacetic acid, 1.5 (to pH 7.0 with 4M KOH); $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot 2H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.12; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4 \cdot 7H_2$), 0.18; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4) \cdot 12H_2O$, 0.02; H_3BO_3, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.11; and $NiCl_2 \cdot 6H_2O$, 0.24. The stock solutions of trace vitamins contained, in milligrams per liter of distilled water, the following: biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pentothenate, 5; vitamin B_{12}, 0.1; p-aminobenzoic acid, 5; lipoic acid, 5; and 2-mercaptoethanesulfonic acid, 100. The salts stock solution contained, in grams per 100 ml distilled water, the following: $(NH_4)_2SO_4$, 3.0; NaCl, 6.0; $MgSO_4 \cdot 7H_2O$, 1.3; $CaCl_2$, 0.06; and KH_2PO_4, 3.0. The phosphate

stock solution contained 3.0 g K_2HPO_4 per 10 ml distilled water. The iron stock solution contained 0.02 g $Fe(SO_4)_2 \cdot 7H_2O$ per 10 ml distilled water. The resazurin stock solution contained 0.5 mg of resazurin per ml of distilled water.

The medium for Mb. thermoautotrophicum was composed of the following: trace minerals solution, 1 ml; salts solution, 10 ml; phosphate solution, 1 ml; $(NH_4)_2SO_4$, 2.7 g; resazurin solution, 1 ml; trace vitamins solution, 10 ml; iron solution, 1 ml; $NaHCO_3$, 5.0 g; cysteine hydrochloride$\cdot H_2O$, 0.5 g; and distilled water to 1000 ml. The final gas phase was 80% H_2-20% Co_2. The pH was 7.2.

The medium for Ms. hungatei was composed of the following: trace minerals stock solution, 1 ml; salts solution, 10 ml; phosphate solution, 1 ml; sodium acetate, 2.5 g; sodium formate, 2.5 g; yeast extract (Difco), 2.0 g; Trypticase (BBL), 2.0 g; resazurin solution, 1 ml; trace vitamins solution, 10 ml; iron solution, 1 ml; $NaHCO_3$, 5.0 g; cysteine hydrochloride$\cdot H_2O$, 0.5 g; and distilled water to 1000 ml. The final gas phase was 80% H_2-20% CO_2. The pH was 7.0.

The medium for Desulfovibrio sp. strain G-11 was composed of the following: trace minerals solution, 1 ml; salts solution, 10 ml; phosphate solution, 1 ml; sodium acetate$\cdot 3H_2O$, 0.68 g; sodium sulfate, 2.84 g; sodium formate, 2.0 g; Trypicase (BBL), 2.0 g; resazurin solution, 1 ml; trace vitamins solution, 10 ml; iron solution, 1 ml; $NaHCO_3$, 5.0 g; cysteine hydrochloride$\cdot H_2O$, 0.5g; and distilled water to 1000 ml. The final gas phase was 80% H_2-20% CO_2. The pH was 7.0.

Escherichia coli was incubated statically at 37°C in 200 ml of the medium in a stoppered 500-ml serum bottle. The medium for E. coli

was Luria broth, which was composed of yeast extract, 5 g; Bacto-tryptone (Difco), 10 g; NaCl, 5 g; glucose, 1 g; and distilled water to 1000 ml. The final gas phase was N_2. The pH was 6.8.

Agar Noble (Difco) was added, final concentration 1.5%, when a solid medium was required. All of the media were sterilized by autoclaving for 20 minutes at 121°C.

Preparation of Butyrate Medium

The control medium for butyrate enrichments and butyrate studies was composed of the following: trace minerals solution, 1 ml; salts solution, 10 ml; phosphate solution, 1 ml; resazurin solution, 1 ml; clarified digester fluid, 50 ml; $NaHCO_3$, 5.0 g; and cysteine hydrochloride$\cdot H_2O$, 0.5 g. The gas phase was 80% N_2-20%CO_2. The pH was 7.2 to 7.4. After the control medium was dispensed to several serum tubes, sodium n-butyrate (Pfaltz and Bauer, Inc., Stamford, Conn.) was added to the remaining control medium to yield a final concentration of 0.3%. This medium was then dispensed, stoppered, and autoclaved for 20 minutes at 121°C .

Anaerobic Techniques

Principles of anaerobic techniques, as described by Hungate (14), were utilized to prepare the media—except Luria broth—and during experimental procedures. To prepare the media, all components, except trace vitamins, $NaHCO_3$, cysteine hydrochloride$\cdot H_2O$, clarified digester fluid, and butyrate, were added to distilled water in a round-bottom flask, boiled for several minutes, and cooled to room temperature in an ice bath while being sparged with 80% N_2-20% CO_2. When the components

were cool, the remainder of the components were added in accordance to the medium being prepared. After the pH was adjusted, if necessary, the media were dispensed into serum tubes (Bellco Glass Co., Vineland, N.J.) or serum bottles (Wheaton Scientific, Millville, N.J.) in which the gas phase had been replaced with 80% H_2-20% CO_2 or 80% N_2-20% CO_2. The serum tubes or serum bottles were closed with butyl rubber stoppers (Bellco No. 2048-11800) which were held in place by crimped aluminum seals (Wheaton No. 224193).

Gas mixtures were purchased from Matheson (Morrow, Ga.) and trace oxygen was removed by passing these gases over heated (350°C) copper turnings.

Transfers of cultures were made with sterile needle and syringe units that had been made anoxic by aspirating sterile reduced medium or sterile anoxic gas.

Prior to the use of all media, except Luria broth, a volume of 1.25% $Na_2S \cdot 9H_2O$ was injected into the media so that the sodium sulfide was diluted 1:50.

Luria broth was prepared and sparged with N_2 gas before being stoppered and sealed into 500 ml serum bottles (Wheaton).

Descriptions of Ecosystems Studied

Thermophilic and mesophilic digesters served as sources of inoculum for various studies. The digesters were similar in design and operation with the exception of source of initial inoculum and incubation temperature. The thermophilic digester was maintained at 55°C whereas the mesophilic digester was maintained at 40°C. The digesters were constructed from aspirator bottles and were stirred semicontinuously. Each

day they received 16 g of feed consisting of 75% bermuda grass and 25% Universal cattle feed (Seminole Brands). The hydraulic detention times were 20 days.

Freshwater sediment samples were taken at Bivens Arm, a eutrophic lake located near the University of Florida. Marine sediment samples were taken from Halodule sp. and Thalassia sp. seagrass beds at Seahorse Key, located near Cedar Key, Florida. Hypersaline sediment samples were taken from Great Salt Lake, Utah, and salterns, from San Francisco Bay, California.

Descriptions of Butyrate Enrichments

Enrichments from thermophilic and mesophilic digesters were begun by placing sludge from those digesters into butyrate medium. Enrichments from Bivens Arm were initiated by placing sediments into butyrate medium. Enrichments with marine sediments were begun by using sulfate-free artificial seawater (SF-ASW) instead of distilled water as given in the description for butyrate medium. The SF-ASW was composed, in grams per liter of distilled water, of the following: NaCl, 21.15; $MgCl_2 \cdot 6H_2O$, 9.65; $CaCl_2$, 1.0; NH_4Cl, 0.25; KCl, 0.5; KBr, 0.086; $SrCl_2 \cdot 6H_2O$, 0.022; and H_3BO_3, 0.023. Enrichments with hypersaline sediments were begun by adding sodium n-butyrate, final concentration 0.3%, to the hypersaline sediments.

Thermophilic Coculture Isolation

A stable thermophilic enrichment was used as a source of inoculum for coculture isolation attempts. The enrichment was serially diluted in the control medium, and roll tubes were prepared from these dilutions.

Each dilution was a source of inoculum for two butyrate control (BC) roll tubes, as well as for three butyrate experimental (BE) roll tubes which contained 0.3% butyrate. Before the tubes were rolled out, they received 0.5 ml of turbid, active Mb. thermoautotrophicum, which served as the hydrogenotroph. The roll tubes were incubated at 55°C.

Mesophilic Coculture Isolation

A stable mesophilic enrichment was used as a source of inoculum for coculture isolation attempts. The enrichment was serially diluted as described for thermophilic coculture isolation attempts. Also, duplicate BC and triplicate BE roll tubes were prepared at the appropriate dilutions. Desulfovibrio sp. strain G-11, 0.5 ml of active culture per roll tube, was used as the hydrogenotroph. These roll tubes were incubated at 37°C.

Gas Chromatography Methods

Methane concentrations were measured by use of a Hewlett-Packard model 5880A gas chromatograph. Gases were separated in a 1.8-m by 1.0-mm stainless steel column packed with Carbosphere mesh 80/100 (Alltech Associates, Inc., Deerfield, Ill.), and were measured with a thermal conductivity detector. Helium was the carrier gas. Column and detector temperatures were maintained at 130 and 145°C, respectively. Methane concentrations were determined by comparison to standards (Ultra High Purity Methane, Matheson). Gas pressures in stoppered vessels were determined by use of a pressure transducer (Setra System, Inc., Acton, Mass.).

Volatile fatty acids (VFAs) were measured by use of a Hewlett-Packard 5880A gas chromatograph. They were separated in a 1.8-m by

1.0-mm glass column packed with 8% SP1000, 2% SP1200, and 1.5% H_3PO_4 on 80/100 mesh Chromosorb W AW 8100 (Supelco, Bellefonte, Pa.), and were measured with a flame ionization detector. Helium was the carrier gas. Injector, oven, and detector temperatures were 145, 130, and 175°C, respectively. Samples were mixed with an equal volume of 4% o-phosphoric acid, centrifuged at 12,800 x g for 2 minute (22°C), and the supernatant was frozen until VFA determinations were made. Each VFA concentration was determined by comparison to standards.

Preparation and Use of E. coli Membrane Fragments To Attempt Isolation of Pure Cultures of Hydrogenogenic Bacteria

Statically grown E. coli were centrifuged at 5,000 x g for 10 minutes (5°C) and washed twice with buffer. The buffer was composed of the following: NaCl, 0.4M; $MgSO_4 \cdot 7H_2O$, 0.02M; and KH_2PO_4, 0.1 M. The pH was 7.0. Cells and buffer were prechilled to 5°C. Membrane fragments were prepared by passing the cell suspension twice through a French pressure cell at 20,000 psi. The lysate was centrifuged at 5,000 x g for 10 minutes (5°C), and the supernatant was frozen at -20°C until used.

The lysate was sterilized by being passed through a sterile 0.2-μm membrane filter. Fumarate, final concentration 20 mM, was filter sterilized (Falcon 7103) and aseptically added to the BC or BE medium. The source of inoculum was the stable mesophilic butyrate enrichment. Anaerobic roll tubes were prepared so that they contained lysate and fumarate, lysate or fumarate, or no addition. The roll tubes were incubated at 37°C.

Microscopy and Photomicroscopy

A Carl Zeiss Standard WL microscope equipped for epifluorescence was used for observation of wet mounts and photomicroscopy. Light of the 420-nm wavelength was provided by a mercury light source (HBO 50 DC 3) and a filter set comprised of an exciter filter (BP 390-440), a chromatic beam splitter (FT 460), and a barrier filter (LP 475). A Leica camera back was attached to the microscope for photomicroscopy. Kodak Technical Pan film 2415 was exposed for times in accordance to previously exposed test rolls. The film was developed according to Kodak instructions and printed on Kodak F5 RC or Polycontrast RC paper.

RESULTS

Production of Methane When Various Ecosystems Were Enriched with Butyrate

When various ecosystems were enriched with butyrate, not all produced methane (Table 3). Anaerobic digesters and freshwater sediments produced methane with little or no lag, whereas marine sediments in sulfate-free artificial seawater required about 2 weeks for methane production to begin. Methane was not produced after several months of incubation when hypersaline sediments were enriched with butyrate.

Description of Thermophilic Butyrate Enrichments

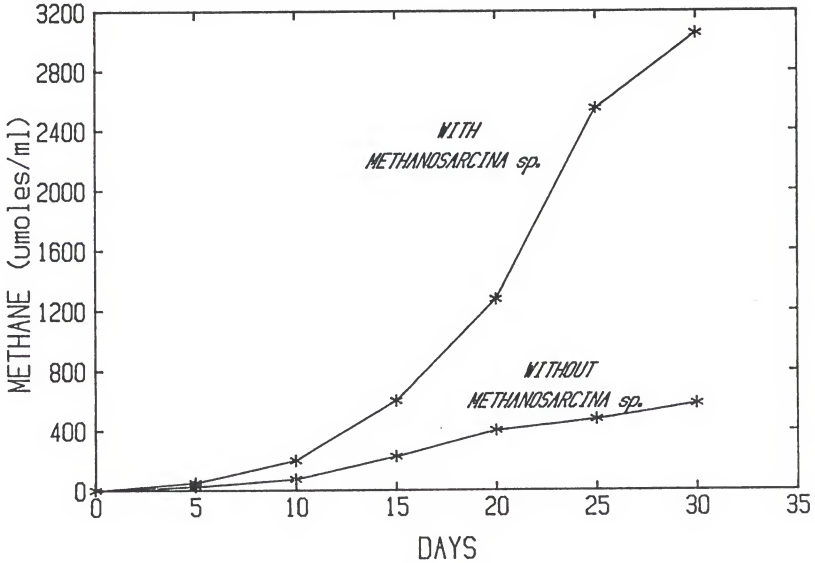
The population of butyrate-utilizing bacteria in a 55°C digester was enumerated by the 5-tube most-probable-number (MPN) method. After a 4-week incubation period, 4.5×10^6 butyrate-utilizing bacteria per ml sludge were found. The lower dilution MPN tubes produced significantly more methane than did the higher dilution MPN tubes. Examination revealed that the lower dilution MPN tubes contained a Methanosarcina sp. Butyrate enrichments were established by use of each of these distinct dilution types as an inoculum. The greater methane production by the enrichment containing the Methanosarcina sp. is shown in Figure 2. Acetate accumulated in the enrichment without the Methanosarcina sp. but disappeared in the enrichment with the Methanosarcina sp. Butyrate was utilized by both enrichments. The Methanosarcina sp. was isolated and would not grow alone when H_2-CO_2 was the only methanogenic substrate

Table 3. Examination of various ecosystems for methane production from butyrate enrichments

Source of enrichment	Methane produced[a]	Lag period before onset of methane production
Thermophilic digester	Yes	None
Mesophilic digester	Yes	None
Bivens Arm	Yes	3 days
Halodule, sp. seagrass bed	Yes	14 days
Thalassia, sp. seagrass bed	Yes	14 days
Great Salt Lake	No	
San Franscisco Bay saltern	No	

[a]Indicates production of methane in medium with butyrate minus methane production in medium without butyrate.

Figure 2. Methane production by thermophilic butyrate-utilizing enrichments with and without Methanosarcina sp. present.

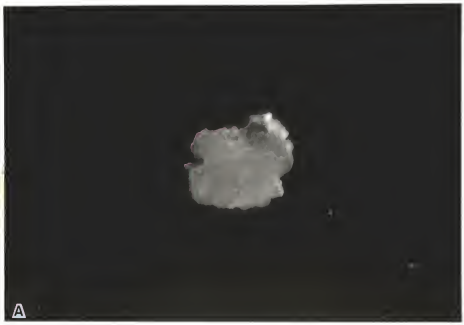

3200
2800
2400
2000
1600
1200
800
400
0
METHANE (umoles/ml)
0
5
10
15
20
25
30
35
DAYS
WITH
METHANOSARCINA sp.
WITHOUT
METHANOSARCINA sp.

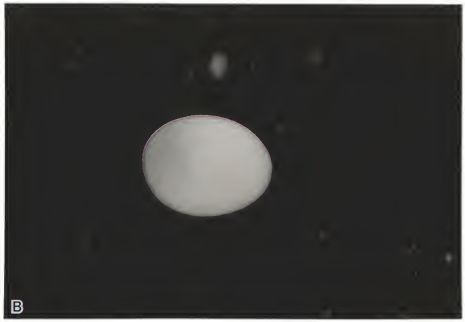
present, growing only in the presence of acetate. This organism was tentatively identified as Methanosarcina strain TM-1 (35). Both enrichments contained rod-shaped bacteria that autofluoresced under examination by 420 nm epifluorescence microscopy, indicating the presence of Factor F420 found in methanogens (10,25). This methanogenic rod-shaped bacterium, tentatively identified as a strain of Methanobacterium thermoautotrophicum, utilized H_2-CO_2 for growth. There were several other rod-shaped nonfluorescing bacteria present. Therefore, it was difficult to know which bacterium utilized butyrate. The enrichments were transferred every 7 to 10 days after being analyzed and were found positive for methane production. After 4 months, a stable enrichment was obtained. A stable enrichment was defined as having a few morphotypes present consistently. M. thermoautotrophicum was always present as the largest population of bacteria, generally comprising 90% of the bacteria in each microscopic field.

Isolation of Thermophilic Butyrate-Utilizing Cocultures

The stable thermophilic enrichment was used as a source of inoculum for attempts to isolate butyrate-utilizing cocultures. M. thermoautotrophicum was used as a hydrogenotrophic partner. Anaerobic roll tubes were incubated for about 4 weeks until methane was detected in the gas phase and colonies appeared. Basically, two colony types were present. One colony type was brownish, granular, and irregular in shape; averaged 2 mm in diameter (Figure 3(A)); and autofluoresced when exposed to 420 nm light. This colony resembled Methanosarcina TM-1, but, when a wet mount of the colony was examined, it was found to be composed of the

Figure 3. Photomicrographs of thermophilic coculture colony types. (A) Colony type that contains Methanosarcina sp., Methanobacterium thermoautotrophicum, and a curved rod. (B) Colony type that contains Mb. thermoautotrophicum and a curved rod.


A


B

Methanosarcina sp., Mb. thermoautotrophicum, and a nonfluorescing, rod-shaped bacterium. The second colony type (Figure 3(B)) was white and circular with an entire margin, average 1 mm in diameter, and autofluoresced when exposed to 420 nm light. Upon microscopic examination, the colony was found to be composed of Mb. thermoautotrophicum and a nonfluorescing rod-shaped bacterium. The two colony types were found to be predominately composed of Mb. thermoautotrophicum. The colonies appeared only in the BE medium and not in the BC medium.

A photomicrograph of the coculture is shown in Figure 4. The Methanosarcina sp. is not shown in this photomicrograph. Mb. thermoautotrophicum, as shown in the photomicrograph, was rod shaped, autofluoresced at 420 nm, and had greater contrast than the butyrate-utilizing bacterium. Several examples are indicated by the single arrows. The butyrate-utilizing bacterium was a slightly curved, gram-negative rod that averaged 2 to 3 μm in length, occurred singly, was nonmotile, and did not contain spores. Several examples are indicated by the double arrows in Figure 4.

The colonies were picked and placed into fresh medium and were rolled out. Once colonies appeared again, they were picked and placed onto slants prepared in serum tubes. After 6 weeks methane was not present in the gas phase and growth had not occurred. The enrichment was rolled out again, and once colonies appeared they were picked and placed onto slants. Again, methane was not produced and growth did not occur. The entire process was repeated with the same results. The colonies were viable because they could be placed into liquid medium and would grow. Because this culture only produced two colony types that

Figure 4. Photomicrograph showing Methanobacterium thermoautotrophicum, indicated by a single arrow, and the butyrate-utilizing bacterium, indicated by the double arrows.


5 µm

could not be routinely grown on slants, it should probably be referred to as a highly purified enrichment. For the sake of brevity, however, it is referred to as a coculture. The coculture was maintained on slants by the injection of active enrichments onto the slants.

Studies on the Thermophilic Coculture

The coculture was incubated at various temperatures to determine whether the butyrate-utilizing coculture was capable of growth at other temperatures. Table 4 shows the differing temperatures tested and the results. When Methanospirillum hungatei, a mesophilic, hydrogen-utilizing methanogen, was added to the coculture, only trace amounts of methane were formed at 37°C. The amount of methane formed was less than 5% of that formed when the coculture was incubated at 55°C. Methane was not produced when the coculture was incubated at 45 or 70°C.

The coculture was examined to see whether clarified digester fluid (CDF) could be replaced by rumen fluid (RF) or deleted from the medium. Table 5 shows that when neither CDF nor RF was a component of the medium, methane production was greatly diminished. The addition of rumen fluid did not stimulate methane production and was inhibitory at concentrations of 20% and above on day 17. Clarified digester fluid addition resulted in consistent methane production at all concentrations tested. When the basal medium without butyrate (BE) contained RF, methane was produced in greater quantities as compared to when the BC medium contained CDF. This result indicated that greater quantities of methanogenic substrates were present in the RF. The quantity of methanogenic substrates in the media containing 20 and 30% RF inhibited the production of methane from butyrate (Table 5).

Table 4. Effects of various temperatures on the methane production by a thermophilic butyrate-utilizing coculture

		Methane production (μmoles)[a]				
		Day 2	Day 5	Day 8	Day 13	Day 26
37°C[b]	A	0.87 ± 0.6	2.4 ± 3.3	4.83 ± 3.02	7.9 ± 5.4	14.4 ± 4.9
	B	MND[c]	MND	MND	MND	MND
	C	1.01	0.99	1.83	1.02	0.96
45°C		MND	MND	MND	MND	MND
55°C		1.07 ± 0.4	2.59 ± 1.6	51.58 ± 10.5	304.7 ± 19.4	298.8 ± 15.0
70°C		MND	MND	MND	MND	MND

[a]Values represent a mean of triplicate determination plus or minus standard deviation, except for M. hungatei alone, where they are single determinations.

[b](A) coculture with M. hungetei; (B) coculture without M. hungatei; (C) M. hungatei alone.

[c]MND equals methane not detected.

Table 5. Effects of various concentrations of rumen fluid (RF) or clarified digester fluid (CDF) on the percentage of methane production by thermophilic coculture

	Methane production (%)		
	Day 3	Day 9	Day 17
No addition	0	1.10 ± 0.02[a]	6.25 ± 0.42
Rumen fluid (RF)			
5%	LTC[b]	3.63 ± 2.57	38.11 ± 10.57
10%	LTC	LTC	41.30 ± 0.46
20%	LTC	LTC	24.00 ± 4.62
30%	LTC	LTC	LTC
Clarified digester fluid (CDF)			
5%	LTC	7.24 ± 1.1	42.87 ± 2.25
10%	LTC	8.16 ± 1.2	41.34 ± 2.00
20%	LTC	9.38 ± 0.7	40.04 ± 3.27
30%	LTC	8.03 ± 3.1	36.61 ± 3.36
Basal medium without butyrate			
RF	ND[c]	9.80 ± 2.2	22.62 ± 11.45
CDF	ND	3.60 ± 1.1	5.10 ± 1.69

[a]Mean of three tubes plus or minus standard deviation.

[b]Less than control.

[c]Not determined.

The effect of the addition of 0.1% yeast extract to the thermophilic coculture is shown in Table 6. The addition of yeast extract resulted in a 142% increase in methane produced after 18 days' incubation by the coculture containing the acetate-utilizing Methanosarcina sp. At day 22, the increase in methane production by the coculture with the Methanosarcina sp. was 28%. Yeast extract did not stimulate methane production and showed a slight inhibition of methane production in the coculture when the Methanosarcina sp. was absent.

Antibiotics known to affect cell wall synthesis of eubacteria but not archaebacteria were added to the coculture. Figure 5 shows that penicillin G (3000 U/ml) and D-cycloserine (0.1 mg/ml) caused the complete inhibition of methane production by the coculture.

The presence of hydrogen in the gas phase (80% H_2-20% CO_2) inhibited the utilization of butyrate by the coculture (Figure 6). The gas phase was replaced every 2 days until day 8. At that time, indicated by the arrow, hydrogen was not detected in the gas phase because of its removal by Mb. thermoautotrophicum. The culture was allowed to continue incubating in the absence of hydrogen to determine if the butyrate utilizers had been killed or merely inhibited by the hydrogen. After a lag period, butyrate utilization began with the butyrate being rapidly utilized (Figure 6).

In general, the enrichments utilized butyrate faster and produced methane quicker when they were incubated without shaking. When the enrichments were shaken, methane production showed a longer lag period, but if the shaking was stopped, methane production increased.

Table 6. Effects of the addition of 0.1% yeast extract to thermophilic butyrate-utilizing enrichments

	Methane production (μmoles)[a]			
	Without *Methanosarcina*		With *Methanosarcina*	
	-YE	+YE	-YE	+YE
Day 2	0	0	0	0
Day 11	0.9	1.8	1.0	0.1
Day 18	36.9	54.6	99.4	240.8
Day 22	62.1	53.5	244.4	313.3

[a]Values represent means of duplicate tubes.

Figure 5. The effect of eubacterial antibiotics on methane production by a thermophilic butyrate-utilizing coculture. The antibiotics were penicillin G (3000 U/ml) and D-cycloserine (0.1 mg/ml).

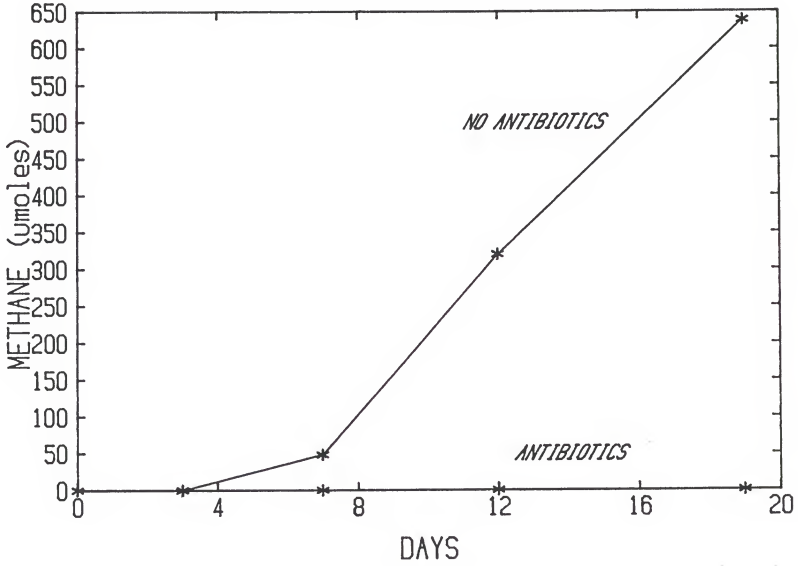
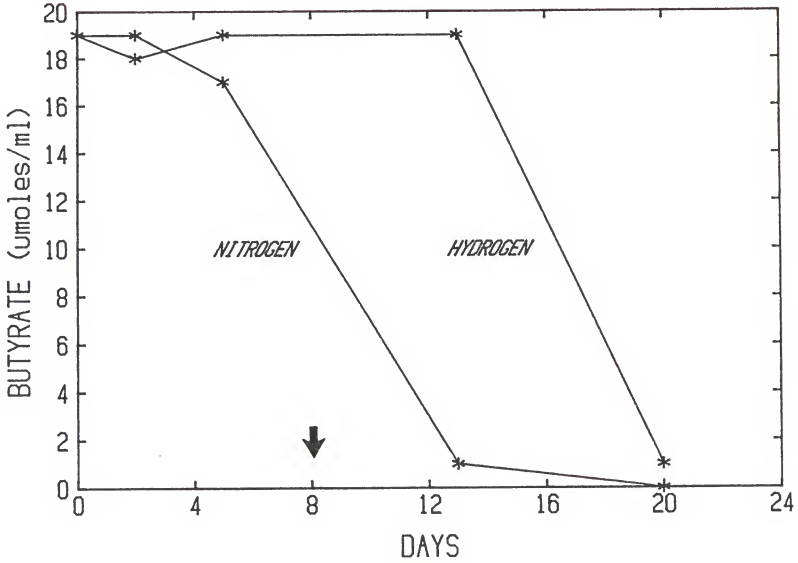

METHANE (umoles)
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0
4
8
12
16
20
DAYS
NO ANTIBIOTICS
ANTIBIOTICS

Figure 6. The effect of hydrogen in the gas phase on butyrate utilization by a thermophilic butyrate-utilizing coculture. The hydrogen was replaced every 2 days until day 8.


BUTYRATE (umoles/ml)
DAYS
NITROGEN
HYDROGEN
0
2
4
6
8
10
12
14
16
18
20
0
4
8
12
16
20
24

Effects of Pumping Butyrate into a Thermophilic Digester

Volatile fatty acids were found in low concentrations in the thermophilic digester. Acetate concentration was 3 to 4 μmoles/ml of sludge, whereas n-butyrate concentration was less than 0.5 μmoles/ml of sludge. A stock solution of sodium n-butyrate was prepared so that it could be pumped into the digester at the rate of 10 μmoles/ml of sludge per day. Figure 7 shows the theoretical accumulation of butyrate if it were not utilized by the digester and the actual concentrations measured. Butyrate did not accumulate in the digester when pumped at this concentration. The concentrations of acetate and propionate are shown in Figure 8. Acetate concentration increased from about 3 μmoles/ml of sludge to about 35 μmoles/ml of sludge by day 23. Propionate concentration increased to about 3.3 μmoles/ml of sludge by day 23. The ratio of gas produced by the butyrate-amended digester versus the control digester was initially 1.5 and by day 23 had stabilized at about 1.4 (Figure 9). The percentage of methane in the gas phase increased from 58% to 68%. The pH increased from 7.3 to 7.8, where it remained stable.

The digester maintained consistent levels of acetate (33 to 35 μmoles/ml of sludge), butyrate (0.7 to 0.75 μmoles/ml of sludge), and ratio of gas production (1.36 to 1.37) from day 18 to day 23. These levels indicated that a stable digestion had been attained and that the rate of addition of butyrate was not exceeding the capability of the digester for utilization of butyrate. To determine the concentration of butyrate that would have to be infused into the digester in order to exceed the ability of the digester to maintain a stable digestion, the rate of addition would need to be increased. Therefore, on day 24, as indicated

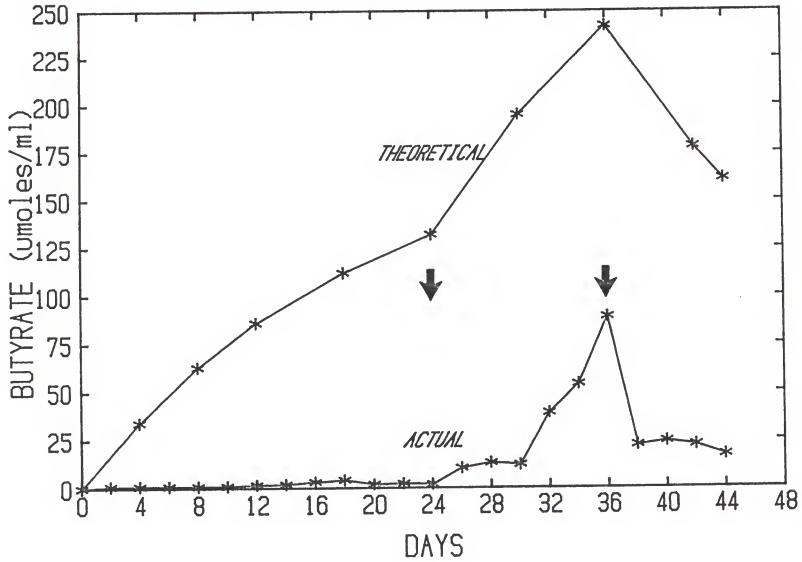

BUTYRATE (umoles/ml)
250
225
200
175
150
125
100
75
50
25
0
0
4
8
12
16
20
24
28
32
36
40
44
48
DAYS
THEORETICAL
ACTUAL

Figure 8. Concentration of acetate and propionate in a thermophilic digester supplemented with butyrate. See legend for Figure 7 for conditions.

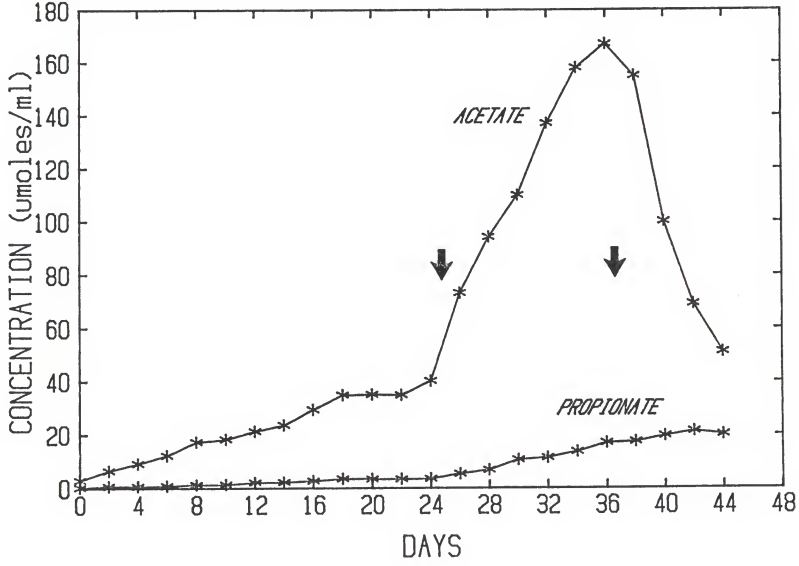
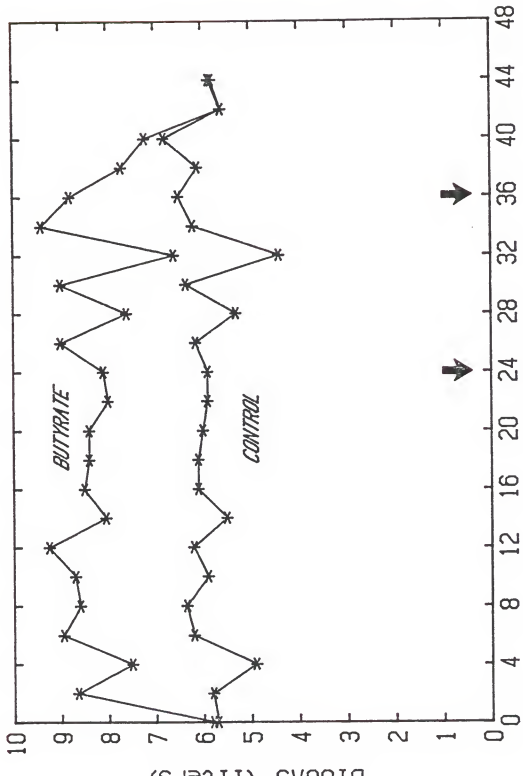

CONCENTRATION (umoles/ml)
DAYS
ACETATE
PROPIONATE
0
20
40
60
80
100
120
140
160
180
0
4
8
12
16
20
24
28
32
36
40
44
48

Figure 9. Biogas production by a thermophilic digester supplemented with butyrate. See legend for Figure 7 for conditions.


BIOGAS (liters)
DAYS
BUTYRATE
CONTROL
0
1
2
3
4
5
6
7
8
9
10
0
4
8
12
16
20
24
28
32
36
40
44
48

by the arrows in Figures 5, 7, and 8, the concentration of sodium n-butyrate infused into the digester was doubled so that the rate of addition would be 20 μmoles butyrate/ml of sludge per day. The levels of all VFAs began to increase. By day 36 the concentrations, in μmoles/ml, were acetate, 167; propionate 16.9; and n-butyrate, 8.9. During this increase of VFAs, the biogas production ratio remained between 1.4 and 1.5 and on day 36 was 1.35. Because of the rapid increase in VFAs, the addition of butyrate was stopped.

Enrichments from Mesophilic Ecosystems

Butyrate enrichments were begun with a mesophilic, 40°C digester as the source of inoculum. These enrichments were analyzed each week for methane production, and the enrichments that produced the greatest quantities of methane were transferred to fresh medium. After about 8 transfers the enrichment was analyzed for VFA concentrations. The acetate concentration was 17.2 μmoles/ml (acetate not detected in uninoculated medium), whereas the butyrate concentration was 0.16 μmoles/ml (about 22 μmoles/ml in uninoculated medium). Microscopic examination showed that the predominant bacterium was an irregular coccus that autofluoresced when exposed to 420 nm of light. A bacterium resembling Methanosarcina barkeri was also observed. In addition, nonfluorescing, short, nonmotile, curved-rod shaped and long, nonmotile, rod-shaped bacteria were observed.

Sediments from a eutrophic freshwater lake, Bivens Arm, were used to begin butyrate enrichments. After weekly transfers for several months, the enrichments were examined by phase-contrast microscopy. The bacteria in the most predominant numbers were Methanospirillum hungatei

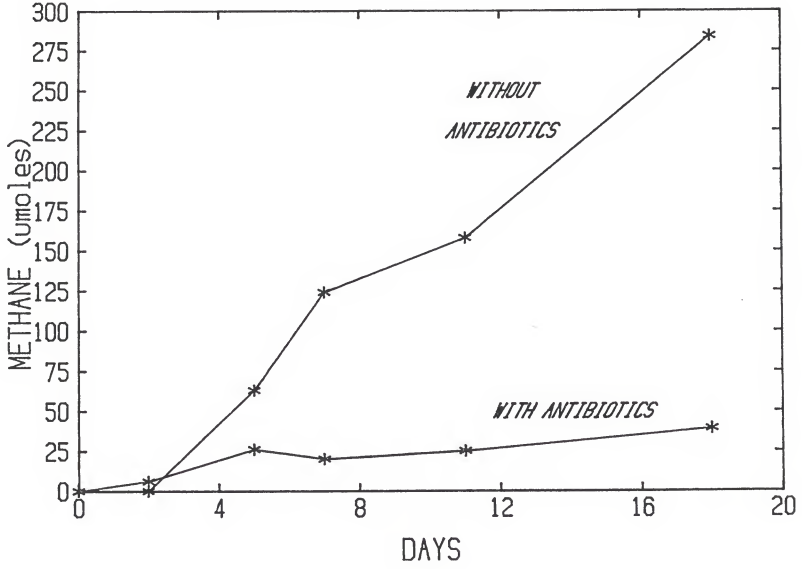
and a Methanococcus sp. There were also small numbers of Methanosarcina barkeri present. In addition, there were nonfluorescing long rods and short curved rods present.

Eubacterial antibiotics were added to mesophilic sludge enriched with butyrate. In the enrichment without antibiotics, about 300 µmoles of methane were found in the gas phase, whereas when antibiotics were present, the gas phase contained only about 40 µmoles of methane (Figure 10). The effect of the antibiotics was not on the H_2-CO_2-utilizing methanogens because methane was formed in the presence of the antibiotics when 80% H_2-20% CO_2 was the gas phase. All of the butyrate in the enrichment without antibiotics was utilized, whereas 87% of the butyrate remained in the enrichment with antibiotics.

Isolation of Mesophilic Butyrate-Utilizing Cocultures

A stable mesophilic enrichment was the source of inoculum for attempts to isolate butyrate-utilizing cocultures. Desulfovibrio G-11 was used as the hydrogenotrophic partner. After 28 days of incubation at 37°C, colonies with blackened centers appeared in roll tubes with butyrate and sulfate, but not in roll tubes with butyrate alone or sulfate alone. Also, colonies which fluoresced when exposed to 420 nm of light were observed. Both types of colonies were picked from the roll tubes and placed onto slants of butyrate-containing medium. The fluorescent colonies grew within 10 days on the slants and produced methane. However, there were four morphotypes of bacteria present. Therefore, this culture was rolled out again in an attempt to isolate colonies composed of two members. The black-centered colonies produced visible growth on slants and were transferred to liquid broth for further study.

Figure 10. Effect of eubacterial antibiotics on methane production by butyrate enrichments from a mesophilic digester. The antibiotics were penicillin G (3000 U/ml) and D-cycloserine (0.1 mg/ml).


300
275
250
225
200
175
150
125
100
75
50
25
0
METHANE (umoles)
0
4
8
12
16
20
DAYS
WITHOUT
ANTIBIOTICS
WITH ANTIBIOTICS

Attempts To Isolate Butyrate-Utilizing Hydrogenogens in Pure Culture with E. coli Membrane Fragments

E. Coli were statically grown for 6 hours at which time hydrogen was present in the gas phase. The lysate from French Pressure-cell passage was filter sterilized and injected into molten agar tubes containing stable mesophilic enrichment. The amount injected was 0.1 ml (57.0 ± 1.6 mg dry weight per ml). The roll tubes were incubated at 37°C. After about 4 weeks, colonies were present in the tubes containing butyrate and E. coli membrane fragments and fumarate. These colonies were not present in tubes with butyrate alone or E. coli membrane fragments alone. The colonies were white, circular with entire margins, and 1 to 2 mm in diameter.

DISCUSSION

Organic matter is degraded exclusively to methane and carbon dioxide in nongastrointestinal ecosystems where light, nitrate, oxygen, and sulfate are absent. This degradation requires at least three groups of bacteria (21,29): fermentative, hydrogenogenic, and methanogenic. The hydrogenogenic bacteria are the least understood of the three groups, with only two species being known (5,22,23).

When several ecosystems were enriched with butyrate, only digesters and freshwater sediments produced methane in a short or no lag period. These ecosystems continually receive organic matter which undergoes degradation to methane. Thus, these ecosystems have a population of butyrate-utilizing bacteria and can readily degrade butyrate to methane. When seagrass beds were enriched with butyrate in sulfate-free artificial seawater, methane was not produced until after a 2-week lag period. The lag in methane production may have resulted from butyrate being utilized by fatty acid-utilizing, sulfate-reducing bacteria (SRB) which were reducing the sulfate that remained in the sediments. These enrichments smelled strongly of H_2S before and after methane production began. Desulfovibrio spp. will degrade lactate (7,20) or ethanol (7) in the absence of sulfate when methanogens are present to remove hydrogen, the electron sink product. In these marine sediments, once sulfate is depleted the methanogens may participate in the degradation of butyrate by removing H_2 produced by SRB. The inability of the microflora

of hypersaline sediments to produce methane when enriched with butyrate indicates that butyrate may not be a typical substrate in the ecosystems examined.

The thermophilic digesters were chosen for more detailed study. Thermophilic digestion may have several advantages over mesophilic digestion. Varel et al. (32) reported the advantage of being able to reduce retention times to less than 6 days at thermophilic (>45°C) temperatures. Also, different microflora of various plant biomasses added to a thermophilic digester would not compete with the digester microflora. If competition were allowed, then it might be possible for destabilization of the digester to occur.

The thermophilic digester had ca. 5×10^6 butyrate-utilizing bacteria/ml of sludge, a finding similar to that in another study (17). When a thermophilic Methanosarcina sp. that utilized acetate, but not H_2-CO_2, was present in butyrate enrichments, greater quantities of methane were produced and the enrichments seemed more stable. The enhanced methane production resulted from the decarboxylation of acetate with the concomitant production of methane by the Methanosarcina sp. Table 2 shows the equation for the oxidation of butyrate which resulted in the production of acetate and hydrogen. The removal of hydrogen made it thermodynamically possible for the reaction to occur and allowed the hydrogenogen to continue metabolic processes because of the recycling of NADH. The additional removal of acetate made the thermodynamics of the equation even more negative, thus enhancing the stability of the enrichments. Thermophilic methanogenic butyrate enrichments were composed primarily of the H_2-utilizing Methanobacterium thermoautotrophicum, with other morphotypes present in smaller numbers. Since M.

thermoautotrophicum was present in large numbers in the butyrate enrichments, it was the bacterium of choice as a hydrogenotrophic partner for coculture isolation attempts. When butyrate enrichments were rolled out with M. thermoautotrophicum, two colony types were observed. When transferred onto slants, the colonies did not grow, possibly because of the change in the oxidation-reduction potential occurring when the serum tubes were opened for inoculation. This lack of growth might be overcome if a reducing agent were used which would maintain a lowered oxidation-reduction potential for a greater length of time.

The two previously described hydrogenogenic bacteria are mesophilic (5,22,23). In order to determine whether the butyrate-utilizing bacterium isolated from the thermophilic digester was in fact a thermophile, the coculture was incubated at several temperatures. The data for this experiment are found in Table 4. When the coculture was incubated at 37°C, methane was not detected. The lack of methane production could possibly have resulted from the inability of M. thermoautotrophicum to grow. Therefore, a mesophilic H_2-CO_2-utilizing methanogen, Methanospirillum hungatei, used in the study of the two reported hydrogenogens (5,22,23), was added to the coculture. If the butyrate-utilizing bacterium could produce H_2 at 37°C. then M. hungatei would oxidize it and methane should be detected. Table 4 shows that a small amount, ca. 14 µmoles, of methane was produced after 26 days of incubation. When the coculture was incubated at 55°C, the temperature of isolation, ca. 300 µmoles of methane were produced by day 13, half the time required for production of 14 µmoles of methane by the coculture with M. hungatei at 37°C. This indicated that the butyrate utilizer was a thermophilic

bacterium and, hence, was different from S. wolfei, a mesophile. In addition, the thermophilic butyrate utilizer was a nonmotile, slightly curved rod, 2 to 3 μm in length, whereas S. wolfei exhibited sluggest motility and was 7 μm in length (22). Therefore it appears that this bacterium is a new species of anaerobic hydrogenogens. It required a growth factor (or factors) that was present in clarified digester fluid (CDF) but not in rumen fluid (RF) (Table 5). The addition of CDF in increasing concentrations resulted in a stimulation of methane production which may have aided in the isolation and maintenance of these bacteria. The addition of 0.1% yeast extract to the cocultures containing Methanosarcina sp. resulted in a 142% increase in methane production after 18 days of incubation. Yeast extract can replace CDF for growth of methanosarcina TM-1 (P. A. Murray and S. H. Zinder, Abstr. Annu. Meet. Am. Soc. Microbiol., 1983, I8, p. 14). It appeared that the addition of yeast extract to medium with CDF resulted in the enhancement of methane production from acetate by the Methanosarcina sp. It may also be of benefit in the isolation and maintenance of the butyrate-utilizing bacteria.

The addition of penicillin and D-cycloserine completely inhibited methane production by the thermophilic coculture (Figure 5). S. wolfei was inhibited by the addition of penicillin and has been shown to possess a peptidoglycan cell wall (22). Since the thermophilic coculture was inhibited by antibiotics that inhibit eubacteria, it appeared that the butyrate-utilizing bacteria were eubacterial and possessed a peptidoglycan call wall like that of S. wolfei.

The presence of 80% hydrogen in the gas phase inhibited butyrate utilization by S. wolfei (22). When the thermophilic coculture was placed under an 80% hydrogen gas phase, butyrate was not utilized

(Figure 6). However, when hydrogen was removed from the thermophilic coculture, butyrate was utilized (Figure 6). Thus it is indicated that hydrogen inhibited but did not kill the thermophilic butyrate-utilizing bacteria.

The addition of butyrate to the thermophilic digester at the rate of 10 μmoles/ml of sludge per day did not result in the accumulation of butyrate. Thus, it is possible that if this digester could be loaded at a higher rate, the result would be the production of a higher concentration of butyrate. The addition of butyrate at the concentration of 20 μmoles/ml of sludge per day resulted in an unstable digestion. Therefore, the maximum concentration of butyrate that could be infused into this digester and still result in a stable digestion was between 10 and 20 μmoles/ml of sludge per day.

Hydrogenogenic bacteria have been isolated only in cocultures with hydrogenotrophic bacteria. In order to better study these bacteria, the isolation of pure cultures is necessary. Because the hydrogenogenic bacteria are inhibited by hydrogen, the concentration of hydrogen must be maintained close to zero. The use of hydrogenase-containing, cell-free systems may make it possible to maintain the lowered concentration of hydrogen. As a test of this possibility, butyrate-utilizing enrichments from mesophilic ecosystems were initiated to provide inoculum for the isolation of cocultures from which a pure culture might be isolated directly or from the enrichments. An active enrichment was diluted and rolled out in media with various additions. These additions were varied to allow for a number of controls. After 21 days of incubation at 37°C, white colonies were found in anaerobic roll tubes that contained E. coli

membrane fragments, fumarate, and butyrate. If any one of these components was not present in the anaerobic roll tubes, the white colonies did not appear. Thus, it appears that the membrane fragments were able to remove H_2 and may be useful in isolating pure cultures of hydrogenogens.

In 1951, Stadtman and Barker (30) reported a highly purified culture that degraded butyrate to acetate and methane. The methane-producing bacterium was named Methanobacterium suboxydans. This culture has since been lost, and attempts to reisolate it have been unsuccessful. In the present study, an experiment was attempted to isolate a butyrate-utilizing methanogen. Because methanogens are capable of growing in the presence of eubacterial antibiotics that act on the cell wall, sludge from a mesophilic digester was enriched with butyrate in the presence and absence of antibiotics. Figure 10 indicates that a small amount of methane was produced in the presence of antibiotics. The methane may have resulted from the inactivation of antibiotics by the high concentration of organic matter in the digester sludge. Such a result does not exclude the possibility that a butyrate-utilizing methanogen existed. However, additional research is needed to determine with certainty whether one did.

Populations of bacteria in microbial communities exist in a variety of symbiotic relationships. The relationship between hydrogenogenic and hydrogenotrophic bacteria is described as syntrophic by several authors (5,18,21,22,23,24). Alexander (2) defines syntrophism as a relationship which entails a bilateral exchange of growth factors. As presently understood, interspecies hydrogen transfer involves the unidirectional

transfer of a substrate. When one organism uses the excretion of a second as a substrate for growth, the relationship is commensalistic (2). Therefore, what is presently referred to as a syntrophic relationship may in fact be a commensalistic one. As more information becomes available, it may be necessary to redefine the relationship that exists between hydrogenogens and hydrogenotrophs.

In summary, several ecosystems were studied to determine their methane-producing capability when enriched with butyrate. A new species of a thermophilic butyrate-utilizing bacterium was established in coculture. This bacterium was a gram-negative, slightly curved rod that measured 2 to 3 μm in length. Most cells occurred singly. Spores were not observed and cells did not exhibit motility. Growth was inhibited by penicillin. Hydrogen inhibited growth but this inhibition was reversed upon the removal of the hydrogen. Growth did not occur at 37°C in the presence of a mesophilic hydrogen-utilizing methanogen. Mesophilic methane-producing enrichments were initiated to isolate cocultures of butyrate-utilizing bacteria and to attempt the isolation of pure cultures of these bacteria. The isolation of pure cultures was not completely successful and is still being attempted.

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BIOGRAPHICAL SKETCH

John Michael Henson, son of John T. and Martha J. Henson, was born on August 11, 1952, in Greer, South Carolina. After graduating from Greer Senior High School in June 1970, he attended Furman University, Greenville, South Carolina. In 1971 he transferred to the University of South Carolina, Columbia, where in August 1975 he received the degree of Bachelor of Science with a major in biology. In 1976 he entered the graduate program at Clemson University, Clemson, South Carolina, and in 1978 received the degree of Master of Science with a major in microbiology. That fall he joined the faculty at Presbyterian College, Clinton, South Carolina, and taught general biology, microbiology, and cell biology. In 1980 he moved to Gainesville, Florida, and entered the graduate program in the Department of Microbiology and Cell Science at the University of Florida.

On January 4, 1975, he married Ellen Adams Dobson. They have two children, Martha Anne ("Marne"), 6, and Jonathan Adams, 16 months. Henson served in the U.S. Naval Reserve from 1972 to 1978 as a field medical service technician attached to the U.S. Marine Corps Reserve.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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